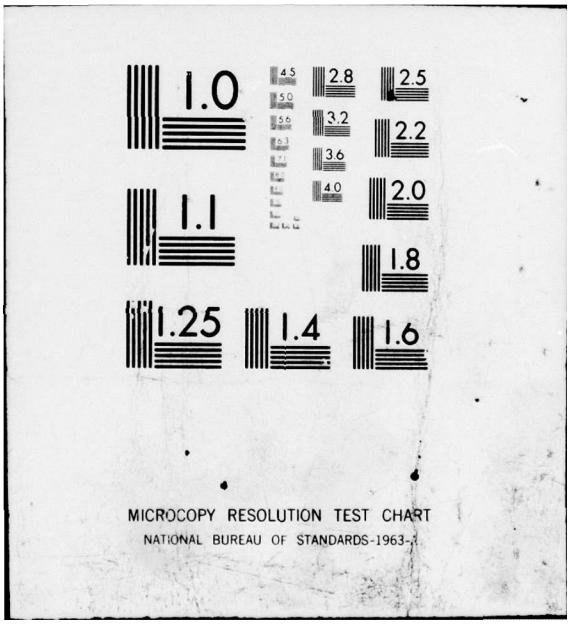
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(6) CHARACTERIZATION AND THE EFFECT OF DRUGS ON PYRUVATE KINASE OF TRYPANOSOMA BRUCEI,

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*This work was supported by the U.S. Army Medical Research & Development Command,
(15) DAMD17-74-C-4140

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ABSTRACT

The following characteristics of T. brucei pyruvate kinase (PK) were examined: pH optimum; temp. optimum; K_m; Mg^{++} requirement; effect of heterotropic modifiers; inhibition by ATP and pyruvate. The effects of some trypanocidal agents on enzyme activity were also noted. T. brucei PK resembles the A isoenzyme in response to amino acids and is sensitive to ATP but insensitive to pyruvate. Inhibition of activity by Hydroxystilbamidine, Berenil (diminazene) and Isometamidium was from 35-50% of controls at 10^{-7} M. With this preparation inhibition by Antrycide (quinapyramine) and Tryparsamide ranged from 5-10% of controls at 10^{-7} M.

Km ; Mg^{++}

10 to the minus 7th power

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African bloodstream trypanosomes of the evansi-brucei subgroups rapidly but incompletely oxidize glucose (Von Brand, 1951) to pyruvate, the major endproduct. Under anaerobic conditions pyruvate and glycerol are produced in equal amounts. The distribution of isotope in pyruvate and glycerol and the amount of $^{14}CO_2$ generated when trypanosomes are incubated with $\left[2,3,4\text{-}^{14}C\right]$ - or $\left[^{14}C_1\right]$ -glucose indicates that the Embden-Meyerhof scheme is the major pathway of glucose utilization in vitro (Grant and Fulton, 1957). The high rate of oxygen consumption during glucose catabolism is attributable to a coupled L-α-glycerophosphate dehydrogenase-oxidase system (Grant and Sargent, 1960). The extremely high glycolytic rate in these organisms prompted a study of pyruvate kinase (PK). In other systems PK is at a control point between glycolysis and gluconeogenesis, necessitating that a negative control be exerted on the enzyme (Seubert, 1971). Feedback inhibition by pyruvate does not appear to occur (Seubert, 1968). At physiologic levels of enzyme, substrate, and heterotropic modifiers there is negligible effect by pyruvate on enzyme activity (Flory, et al, 1974). We report here on the isolation, partial purification and characterization of PK from bloodstream forms of monomorphic T. brucei. We have also examined the effects of some trypanocidal agents on enzyme activity.

MATERIALS AND METHODS

Isolation of trypanosomes: Laboratory mice were infected by the intraperitoneal route (ip.) with 6×10^6 trypanosomes in 0.2 ml TRIS-glucose (TG) buffer, pH 7.4. These animals were the source of the organisms used to infect rats. Male NLR strain (National Lab. Animal Co.,

Creve Cocur, Mo.) were infected with sufficient trypanosomes to yield 8×10^9 organisms/ml blood, within 72 hr. At the peak of parasitemia animals were etherized and bled by cardiac puncture using TRIS-glucose EDTA (TG-EDTA) buffer, pH 7.5. The blood-buffer mixture was centrifuged at 1500 rpm (365xg) for 10 min in an HB-4 rotor (Sorvall). The supernatant was removed and the trypanosomes were gently resuspended in buffer without disturbing the blood layer beneath. The resuspended trypanosomes were removed with minimal contamination, centrifuged at 2500 rpm (1020xg) for 10 min and the supernatant discarded. The pellet was resuspended in TG buffer. This last suspension was freed of blood cells by passage through DEAE-cellulose eluted with TG buffer (Lanham, 1968). By microscopic examination the filtrate contained only trypanosomes. The filtrate was centrifuged at 2500 rpm (1057xg) for 10 min (GS-3 rotor), the supernatant discarded and the pellet resuspended in dist. H_2O as: 1.0 ml packed cell volume: 4 ml dist. H_2O. The cell suspension was sonicated in the cold (Biosinik, macroprobe, setting 60) to achieve 99% breakage, which occurred within 2-3 min, and the sonicate was centrifuged at 15,500 rpm (32,000xg) for 30 min in a SW 27 rotor at 4°C (Beckman L2 Preparative Ultracentrifuge). The supernatant fraction containing PK activity was stored at -19°C until purification.

Streptomycin Sulfate (SMS) Fractionation: Thawed supernatant from the ultracentrifugation step was brought to 2% saturation with SMS, allowed to stand at 0°C for 15 min and centrifuged at 13,000 rpm(22,000xg) in a SM 24 rotor (Sorvall). The supernatant was removed and assayed for protein, specific activity of PK and the presence of contaminating

enzymes (see below). Ammonium Sulfate (AS) Fractionation: The SMS supernatant fraction was brought to 30% AS saturation at 0-4°C, centrifuged at 13,000 rpm (22,000xg) for 10 min, the precipitate resuspended in dist. H_2O and tested for the presence of PK and contaminating enzymes. Sufficient AS was added to the 30% AS supernatant to achieve 40% saturation. Identical centrifugation, resuspension and assay procedures were performed as for the 30% fraction. In accordance with these procedures 45% and 50% fractionations were also performed. Maximal activity was obtained in the 40% saturated resuspended precipitate. Pyruvate Kinase: PK activity was monitored by noting the decrease in absorbance of NADH at 340 nm in a 1.0 cm (path length) cuvette at 27°C on a Cary 15 recording spectrophotometer. The standard assay conditions were: 4.0 mM phosphate buffer, pH 7.5, 8.0 mM $MgSO_4$, 64 mM KCl, 0.14 mM NADH, 0.6 mM phosphoenolpyruvate (PEP), 1.0 mM fructose-1,6-diphosphate (FDP), 1.5 mM ADP, 0.125 units bovine heart lactate dehydrogenase (LDH), and 25 µl of the enzyme preparation per ml of the reaction mixture. L-α-Glycerophosphate Dehydrogenase: L-α-glycerophosphate dehydrogenase activity was monitored by noting the decrease in NADH absorbance at 340 nm. The assay components were: 0.14 mM NADH, 1.5 mM ADP, 1.0 mM FDP, 1.5 mM dihydroxyacetone phosphate (DHAP), 25 µl of the enzyme preparation and 0.8 ml PK mix. NADH Oxidase(s): NADH oxidase activity was determined by utilizing the same assay components as for the PK assay but with deletion of PEP and LDH. PEP Carboxykinase: The presence of PEP carboxykinase was determined by following the disappearance of the enol band at 230 nm at 27°C. The standard assay conditions were: 0.6 mM PEP, 2.5 mM GDP, 1.0 mM FDP, 25 µl enzyme preparation, 50 µl dist. H_2O,

and 0.8 ml PK mix. Gel Chromatography: When significant contamination by L-α-glycerophosphate dehydrogenase was detected, the preparation was further purified on a Sephadex G-100 column using 0.2 M phosphate buffer as eluant. Protein Assays: Protein in supernatants was assayed by the method of Lowry (1951). Protein in the 30% and 40% ammonium sulfate resuspended precipitates, and Sephadex fractions was determined by the Warburg-Christian method (1941).

ENZYME CHARACTERIZATION.

Temperature Optimum: PK activity at 0°C, 10°C, 27°C, and 37°C was determined by the standard assay procedure with all components equilibrated at the designated temperatures. pH Optimum: The pH of the PK mix was adjusted to the desired value with either 0.1N HCI or 0.1N N_aOH. Mg++ Dependency: PK activity at 0, 0.1, 0.3, 1.0, 3.0, 10.0, and 30.0 mM $MgSO_4$ was obtained. The standard assay procedure was used with deletion of $MgSO_4$ from the PK mix and addition of appropriate amounts of $MgSO_4$ solution. Determination of Km: The standard assay procedures was used to determine a Km. The amount of PEP was varied as: 0, 0.01, 0.03, 0.06, 0.10, 0.3, 10, 3.0, 10.0, and 30.0 mM. pH Effect at Varying Substrate Concentrations: PK activity was tested at the following substrate concentrations: 1.0, 1.5, 2.0, 2.5, 3.0, 3.5 mM PEP and at the following pH's: 6.1, 6.3, 6.5, 6.7, 6.9, 7.1, 7.3, and 7.5. Inhibition by ATP: The specific activity of PK in the presence of the following concentrations of ATP was tested: 1.0, 2.0, 3.0, 4.0, and 5.0 mM. Inhibition by Amino Acids: The specific activity of PK in the presence of L-alanine, L-proline, or L-serine (0.5-1.5 mM)

was tested. Endproduct Inhibition: The standard assay was used with the addition of pyruvate as: 0.5, 1.0, 2.0, 3.0, and 4.0 mM. Effect of Trypanocidal Agents: PK was incubated with 10^{-4}, 10^{-5}, 10^{-6} or 10^{-7}M of the following trypanocidal drugs: Berenil (diminazene), Antrycide (quinapyramine), Isometamidium, Hydroxystilbamidine, and Tryparsamide. In these assays a 0.1 mM triethanolamine (TEA) buffer was used; all other constituents were constant.

RESULTS

Maximal PK activity was noted in the 40% as fraction. The preparation showed activity by the major contaminating enzymes (NADH oxidase, L- -glycerophosphate dehydrogenase, and PEP carboxykinase, and was further purified on Sephadex G-100 (Table 1).

Differences in protein concentration between the 30% and 40% fractions was due to the presence of a greater amount of insoluble protein in the former. The 40% fraction represents an ~10-fold purification of the enzyme. The Sephadex fraction represents a 42-fold purification.

The Km for pyruvate kinase was calculated as 1.75×10^{-5}M . The phenomenon of substrate inhibition was noted (Figures 1a, b, and c).

Although PK exhibited maximal activity at pH 6.4 (Figure 2) assays were routinely done at pH 7.5 to provide stability of NADH. The specific activity at pH 7.5 was ~20% of maximum.

The temperature optimum was 27°C (Figure 3). Activity at 0°C, 10°C, and 37°C deviated from the optimal rate by 75%, 58%, and 14%, respectively.

A requirement for magnesium ions was demonstrated (Figure 4). Maximal activity was obtained at ~9.0 mM Mg++.

A pH profile of enzyme activity at varying substrate concentrations was obtained (Figure 5). At lower PEP concentrations maximal activity centered at ~pH 6.4. This optimum shifted to pH 6.7 at the highest concentration tested (3.5 mM).

PK activity was inhibited by ATP in the range, 0.5 to 1.5 mM (Figure 6). Inhibition was immediate and varied from 40% to 64% of control values.

Inhibition by amino acids (heterotropic modifiers) was investigated (Figure 7). L-alanine, L-proline, and L-serine inhibited PK activity. Inhibition by L-alanine (0.5-1.5 mM) was 18-26% of the calculated specific activity of the control. Serine (0.5 mM) and proline (0.5 mM) activated PK activity but not to any appreciable extent. Inhibition by serine (1.0-1.5 mM) and proline (1.0-1.5 mM) ranged from 11-27% and 7-32%, respectively.

Addition of pyruvate revealed both activation and inhibition of activity depending on the concentrations (Figure 8). The inhibitory concentrations were 0.5, 1.0, 2.0, and 3.0 mM for 16, 19, 21, and 2% inhibition, respectively. 4.0 mM pyruvate increased activity by 6%.

A TEA buffer system was used to test the effects of various trypanocidal compounds. This change in buffer system eliminated the problem of drug-phosphate precipitation encountered at even the lowest drug concentrations used. However, precipitation in TEA still occurred at the higher drug concentrations with Isometamidium, Berenil, and Antrycide. Hydroxystilbamidine, Berenil, Tryparsamide, Antrycide and

Isometamidium inhibited PK activity at 10^{-7}M. The order of efficacy was: Hydroxystilbamidine (52-60%), Berenil (42%), Isometamidium (35%), Antrycide (10%), and Tryparsamide (4.9-8.4%) (Table 2). In TEA buffer the specific activity of PK was ~50% of that obtained in phosphate buffer. Addition of drug to the reaction mixture did not affect the pH.

DISCUSSION

T. brucei PK exhibits maximal activity at pH 6.4 and 27°C. Most reported pH optima are at 7.5, the minimal value at which NADH is stable (Bucher and Pfleiderer, 1962). The value of 6.4 is in close agreement with a reported pH optimum of 6.3 for C. fasciculata PK (Marr, 1973).

The temperature optimum for PK has been reported to be 25°C and 37°C. This difference has been attributed to the use of a phosphate buffer and the inclusion of FDP in the reaction mixture (Flory, et al, 1974) in contrast to others where imidazole or TEA buffer was used without FDP (Tanaka, 1967).

Pyruvate kinase binds two moles of PEP per mole of enzyme, (Reynard, et al., 1961). In Table 3 the Km values for various preparations of PK are listed. These values differ considerably even for the same isoenzyme. It would appear that the buffer system, pH and temperature used in these determinations are critical. T. brucei PK activity in 0.1 mM TEA buffer was ~50% of the value obtained in 4.0 mM phosphate buffer. The concentrations of K+ and Mg++ are crucial to the determination of the Km. If the K+ concentration is not at saturating level, Mg++ inhibits PK activity (Rose, et al., 1968). Substrate inhibition

of yeast PK at higher PEP levels (1.0 mM) has been reported (Hess, 1970).

The Km of T. brucei PK, determined from a double reciprocal plot, is 1.75 10^{-5}M (Figure 1b). When a Michaelis-Menten plot was obtained, the Km value increased to 5.0 10^{-4}M (Figure 1a). This difference may be accounted for by the fact that the values for the double reciprocal plot lay below the concentrations at which substrate inhibition occurred (1.0 mM), whereas the Michaelis-Menten plot utilized substrate concentrations in the range, 0.1-30mM.

T. brucei PK requires Mg++ ions for activity. Mg++ plays an essential role in the reaction mechanism and functions as a component of the activie site of muscle pyruvate kinase (Mildvan and Cohn, 1965). A hyperbolic curve is obtained when Mg++ concentration is plotted vs. PK activity, with the maximal velocity occurring at about 8 mM Mg++. The optimal Mg++ range has been reported as 3-8 mM (Carminatti, 1968).

As an endproduct of the PK reaction ATP acts as an isosteric inhibitor. This inhibition is immediate and reversible by increasing PEP or FDP concentrations. These properties are universal for all reported PK's and likewise, T. brucei PK exhibits sensitivity to ATP. However, the physiological significance of this effect is questionable when considered in terms of the dependence by the organism on glycolysis. It seems unlikely that in these organisms ATP would accumulate to an extent necessary to produce inhibition.

PK isoenzymes [L, M, K (or A)], vary in their response to inhibition or activation by amino acids. Both the L and A forms are allosterically by alanine. Only L-alanine inhibits at physiological levels (Carbonell,

1973). The D- and β-alanine forms are without effect, and this differential in specificity would seem to eliminate the possibility that alanine inhibition of the L and A forms is due to the structural similarity between alanine and pyruvate. The marked differences between the responses of the L and M isoenzymes support the view of allosteric rather than isosteric inhibition. Weber, et al., (1968) support the idea of competitive inhibition. Variation in inhibition in the simultaneous presence of known inhibitory amino acids has led to the suggestion of multiple regulatory sites.

Activation of the three forms of PK by various amino acids has been reported. Polar side chains decrease inhibitory potential and serine activates both the A and M forms by approximately 25% (Ibsen and Trippet, 1974).

T. brucei PK resembles the A isoenzyme most closely in its response to amino acids. L-alanine inhibits whereas L-serine and L-proline both inhibit and activate enzyme activity. Activation by serine occurs at 0.5 mM. Serine activation is dependent upon PEP concentration with an inflection point at 0.6 mM, the value used in the present assays. In subsequent experiments variation in the PEP concentration plus increase in serine concentration will be done. Activation by L-proline appears to be unique to T. brucei PK. The possibility of minor contamination of the final enzyme preparation with transaminase and/or decarboxylase activities may mean that the effects noted for the amino acids (where inhibition ranged from as little as 7% to as high as 32%) could be contributed to by competing reactions.

T. brucei PK appears to be insensitive to feedback inhibition by pyruvate. Over a concentration range of 0.5 to 4.0 mM pyruvate, enzymatic activity varied from 80% to 106% of the control value. This absence of feedback may merely reflectactial pyruvate excretion by these organisms.

The effects of several classes of trypanocidal compounds were noted. The four classes examined were: arsenical (Tryparsamide), diamidine (Hydroxystilbamidine and Berenil), phenanthridine (Isometamidium) and quinaldine (Antrycide). It has been demonstrated that the oxidation of keto-acids is a key reaction in arsenical inhibition. (Peters, et al., 1946). This does not appear to be the site of action in trypanosomes which lack α-keto acid oxidases (Ryley, 1955; Grant and Fulton, 1957). Chen (1948) and Marshall (1948) identified the hexokinase reaction as the site of arsenical inhibition. However, Cantrell (1953, 1954) showed that glucose utilization in T. equiperdum was identical in both control and experimental groups. Flynn and Bowman (1974) have reported inhibition of T. brucei and T. rhodesiense PK by melarsen and attribute trypanocidal activity to reaction with intracellular thiol groups.

Tryparsamide (disodium N-phenylglycinamide p-arsenothioglycellate) exhibits moderate trypanocidal activity and is valuable because it passes the blood-brain barrier. In vivo, Tryparsamide is probably reversibly hydrolyzed to arsenoxide and thioglycollic acid (Hawking, 1963). Tryparsamide minimally inhibited T. brucei PK (4.9% and 8.4% at 10^{-7} and 10^{-6}M, respectively). The minimal inhibition noted may be due to conformational and/or structural changes in the enzyme during the purification process.

Two aromatic amidines tested were Hydroxystilbamidine and Berenil. Diamidines appear to be rapidly absorbed by trypanosomes (Girgla-Takla and James, 1974). Berenil has been shown to penetrate the kinetoplast. (Newton, 1967). It has also been suggested that Berenil interferes with carbohydrate metabolism (Bauer, 1958). Both Hydroxystilbamidine and Berenil inhibited PK activity at 10^{-7}M (Table 2). Berenil inhibition suggests an action on carbohydrate metabolism, *in vivo*.

Although isometamidium inhibited PK activity by 55% at 10^{-7}M, generally Phenanthridinium compounds appear to exert their effect only after a latent period of 3-7 cell divisions. It has been postulated that these compounds inhibit the formation of some metabolite necessary for cytokinesis, the delay suggesting the presence of a pool of that metabolite (Hawking, 1963). The mode of interaction between drug and enzyme is unknown.

The *in vivo* mode of action of Antrycide is thought to be similar to that of the phenanthridines. In comparison with other compounds tested, Antrycide exerted minimal inhibitory activity on PK activity, i.e. 10% at 10^{-7}M.

We are currently investigating the nature of the drug-induced inhibitions of enzyme activity and are examining some molecular characteristics of the enzyme protein, e.g. subunit composition. The immunogenic properties of the purified preparation will also be examined.

ACKNOWLEDGEMENTS

We extend our appreciation to Dr. J. Joseph Marr and Dr. Edgar Steck for helpful discussions. We are grateful to the following drug companies who provided generous samples and information on the compounds: Farbwerke Hoechst, A.G. (Berenil); Imperial Chemical Industries, Ltd. (Antrycide); May & Baker Ltd. (Isometamidium, Hydroxystilbamidine).

Table 1

Purification of *T. brucei* Pyruvate Kinase

Preparation	Protein mg/ml	Enzyme Assay	Activity µM PEP/min/ml	Specific Activity µM PEP/min/mg Protein	Total Protein	Total Activity
SW 27 supernatant	4.15	PK	0.57	0.12	80.51	5.0
SMS supernatant	3.75	PK	0.44	0.12	71.25	4.2
		NO	-	-	-	-
30% AS Fraction	0.28	PK	-	-	-	-
		NO	0.61	2.18	4.32	33.8
		LαGPDH	-	-	-	-
		PEPCK	-	-	-	-
40% AS Fraction	0.41	PK	0.17	0.41	5.96	2.5
		NO	-	-	-	-
		LαGPDH	0.39	0.97	5.96	5.8
		PEPCK	-	-	-	-
Sephadex Fraction	0.10	PK	0.57	5.67	0.45	2.6
		NO	-	-	-	-
		LαGPDH	-	-	-	-
		PEPCK	-	-	-	-

Legend = PK = Pyruvate Kinase; NO = NADH oxidase; LαGPDH = L-α-glycerophosphate dehydrogenase; PEPCK = Phosphoenolpyruvate carboxykinase; (-) = no activity; res. = resuspended; AS = ammonium sulfate.

Table 2

Effects of Trypanocidal Compounds on *T. brucei* Pyruvate Kinase

Drug	Concentration, Molarity	Specific Activity μM PEP/min/mg Protein	Percent Inhibition
Hydroxystilbamidine	10^{-7}	0.185 ± 0.007	52
	10^{-6}	0.154 ± 0.001	60
Isometamidium	10^{-7}	0.249 ± 0.016	35
Tryparsamide	10^{-7}	0.366 ± 0.12	4.90
	10^{-6}	0.353 ± 0.04	8.5
Berenil	10^{-7}	0.421 ± 0.116	42.5
Antrycide	10^{-7}	0.633 ± 0.025	10

Table 3

Reported Michaelis-Menten Constants for Pyruvate Kinase

Preparation	Km	Buffer System	pH	Temperature	Ref.
Rabbit Muscle	3.2×10^{-5}	-	8.5	0°C	
Rabbit Muscle	0.7×10	Imidazole	7.5	37°C	
Rat Liver M	0.75×10	Imidazole	7.5	37°C	
Rat Liver L	0.83×10	Imidazole	7.5	37°C	
Rat Liver L	0.15×10	Phosphate	7.5	25°C	
C. fasciculata	0.49×10	Tris	7.3	25°C	
C. fasciculata	0.83×10	Tris	6.3	25°C	
T. brucei	1.75×10	Phosphate	7.5	27°C	

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